The Iron Requirement of *Agrobacterium tumefaciens* for Growth and 3-Ketosucrose Production. The Removal of Iron from Solutions by Seitz Filters

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SUMMARY

Medium for growth of *Agrobacterium tumefaciens* was made iron-deficient by passage through Seitz filter pads, which removed up to 3.2 mg. Fe^{3+} from aqueous FeCl_3 solutions. The growth rate of *A. tumefaciens* in this medium was retarded, no 3-ketosucrose accumulated in the culture supernatant and no glucoside 3-dehydrogenase activity was detected in the cells. Addition of Fe^{3+} to the filtered medium restored the growth rate, glucoside 3-dehydrogenase activity and 3-ketosucrose production. The optimum iron concentrations for maximum glucoside 3-dehydrogenase activity (1 to 3 mg. $\text{Fe}^{2+}/\text{l.}$) and yield of 3-ketosucrose (0.5 mg. $\text{Fe}^{2+}/\text{l.}$) were considerably less than the minimum requirement of 15 mg. $\text{Fe}^{2+}/\text{l.}$ for the maximum growth rate.

INTRODUCTION

Agrobacterium tumefaciens metabolizes disaccharides to the corresponding 3-keto derivatives (Bernaerts & DeLey, 1958; Fukui, Hochster, Durbin, Grebner & Feingold, 1963; Hayano & Fukui, 1967). The enzyme involved has been given the trivial name glucoside 3-dehydrogenase (Hayano & Fukui, 1967). Its specific activity is maximal during the exponential phase of batch growth and decreases as the culture enters the stationary phase (Hayano & Fukui, 1970; A. H. Fensom, W. M. Kurowski & S. J. Pirt, unpublished results). During studies of the factors influencing this loss of activity, some medium was sterilized by Seitz filtration but subsequently supported only slow growth of the bacterium with negligible accumulation of 3-ketosucrose. This was due to the removal of iron from the medium by the filter pads. This effect was exploited to determine the iron requirements of *A. tumefaciens* for normal growth and 3-ketosucrose production. Also, because of the general importance of Seitz filtration in medium preparation, the uptake of iron by the filter pads was investigated quantitatively.

METHODS

Bacterium and growth. *Agrobacterium tumefaciens* NCPPB 396 from the National Collection of Plant Pathogenic Bacteria was maintained on nutrient agar slopes at 4°. Inocula were prepared by removing bacteria from nutrient agar slopes incubated at 30° for 24 to 30 h. and suspending them in 0.9 % (w/v) NaCl. Liquid cultures were incubated at 28° with orbital shaking (200 rev./min.). Bacterial growth was followed turbidimetrically at 520 nm. The medium of McIntire, Peterson & Riker (1942) was modified as follows: ZnSO_4 and NaCl were omitted; KH_2PO_4 (5.34 g./l.) and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (10.86 g./l.) were added and

Fe (1.03 mg./l.) was supplied either as $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5 mg./l.) or as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (5.14 mg./l.) chelated with EDTA (disodium salt, 6.75 mg./l.). Analytical-grade chemicals were used throughout. Iron-deficient medium was prepared by filtering autoclaved medium (100 ml.) containing no added iron through a 6 cm. diameter Seitz filter pad, type HP/EK (Carlson-Ford Sales Ltd, Ashton-under-Lyne, Lancashire).

Assay procedures. Glucoside 3-dehydrogenase activity was estimated from the rate of reduction of the dye, 2,6-dichlorophenol indophenol (DCPIP) using a Unicam SP800 spectrophotometer (Unicam Instruments Ltd, Cambridge). Whole cells were washed twice with 0.1 M-phosphate buffer (pH 7.0) and finally resuspended in a suitable volume of the same buffer. The test cuvette contained 0.1 ml. 2.2 mM-DCPIP; 0.3 ml. M-phosphate buffer (pH 6.04); 0.3 ml. 0.1 M-sucrose; 0.1 ml. cell suspension (2 to 5 mg. dry wt./ml.) and 2.2 ml. distilled water. Sucrose was omitted from the reference cuvette, and the change in extinction was followed at 600 nm. at 20° for 6 min. One unit of enzyme was defined as the amount which oxidized 1 μmole sucrose/min.: this corresponded to the reduction of 1 μmole DCPIP/min.; ϵ for DCPIP = $11.85 \times 10^3 \text{ l. cm.}^{-1} \text{ mol.}^{-1}$ at 600 nm. and pH 6.04 (van Beeumen & DeLey, 1968).

3-Ketosucrose in the culture supernatant was determined by the alkali method of Fukui & Hayano (1969); ϵ for 3-ketosucrose in 0.1 M-NaOH at 340 nm. = $6.5 \times 10^3 \text{ l. cm.}^{-1} \text{ mol.}^{-1}$.

For the determination of iron, 1 ml. of a solution containing not more than 20 $\mu\text{g. Fe}^{2+}$ or Fe^{2+} /ml. was heated with 0.9 ml. 3 M-HCl and 0.1 ml. 0.02 M- KMnO_4 until colourless. After cooling, 2 ml. 2 M-HCl and 2 ml. M-KCNS were added and the extinction at 480 nm. determined after 30 sec. at room temperature.

RESULTS

Iron requirement for normal growth. The growth rate of *Agrobacterium tumefaciens* in Seitz-filtered autoclaved medium was approximately linear whereas in autoclaved medium the rate of growth was exponential (Fig. 1*a*). Cultures darkened on ageing and did not develop a pale yellow pigment as when Seitz filtration was omitted. Filter pads apparently removed iron from the medium as addition of Fe^{2+} (1.03 mg./l.) after filtration completely restored the growth rate of the organism; Zn^{2+} (0.45 mg./l.), Mo^{3+} (0.20 mg./l.), Mn^{2+} (0.25 mg./l.), B^{3+} (0.06 mg./l.) and Co^{2+} (0.12 mg./l.) had no effect. The specific growth rate in Seitz-filtered medium to which iron had been added after filtration was consistently slightly higher than in non-filtered medium containing the same amount of added iron. This may have been due to the filter pads contributing some growth stimulating substance to the medium or else removing some inhibitor, such as a heavy metal, from the medium.

Iron requirement for glucoside 3-dehydrogenase activity. Formation of 3-ketosucrose and glucoside 3-dehydrogenase activity was followed during growth in Seitz-filtered medium and in medium to which Fe^{2+} (1.03 mg./l.) had been added after filtration (Fig. 1). Iron-deficient bacteria had virtually no glucoside 3-dehydrogenase activity and accumulated negligible 3-ketosucrose. With iron-supplemented cultures, as with cultures grown on untreated medium, the specific enzyme activity was maximal during the exponential phase of growth and 3-ketosucrose accumulation reached a peak about 3.5 h. later.

Effect of iron concentration on the specific growth rate, specific glucoside 3-dehydrogenase activity and accumulation of 3-ketosucrose. *Agrobacterium tumefaciens* was grown in media to which were added different amounts of Fe^{2+} chelated with equimolar amounts of EDTA and the specific growth rate, specific activity of glucoside 3-dehydrogenase and accumulation of 3-ketosucrose were followed (Fig. 2). The specific growth rate was maximal at 15 mg.

$\text{Fe}^{2+}/\text{l.}$ whereas maximum specific glucoside 3-dehydrogenase activities were obtained between 1 and 3 mg. $\text{Fe}^{2+}/\text{l.}$, i.e. at iron levels several times lower than that needed for an optimum growth rate. The maximum accumulation of 3-ketosucrose in the culture supernatant occurred at 0.5 mg. $\text{Fe}^{2+}/\text{l.}$ Detection of glucoside 3-dehydrogenase activity in cultures grown on medium with no added iron can be attributed to traces of iron coming from the other components of the medium.

Fig. 1. Growth of *Agrobacterium tumefaciens* in medium made iron-deficient by Seitz filtration (Δ) and in Seitz-filtered medium to which iron (1.03 mg. $\text{Fe}^{2+}/\text{l.}$) was added after filtration (O). (a) Growth curves. (b) Specific cellular glucoside 3-dehydrogenase activity. (c) 3-Ketosucrose in the culture supernatant.

Uptake of iron by Seitz filter pads. Different concentrations of aqueous FeCl_3 solutions (25 ml.) were Seitz-filtered and the concentrations of Fe^{3+} in the filtrates compared with those in the original solutions (Fig. 3). Similar determinations were made with FeSO_4 solutions acidified with conc. H_2SO_4 . With FeCl_3 solutions almost 100 % uptake occurred when amounts of Fe^{3+} less than 1.5 mg. were filtered. With greater amounts of iron the weight of Fe^{3+} absorbed tended towards a plateau value of 3.2 mg. Fe^{3+} which represented the maximum amount of iron that the filter pads were capable of removing from solution. With Fe^{2+} the plateau value was about 0.34 mg. iron per filter pad. This probably represented an uptake of Fe^{2+} because if the loss of iron from FeSO_4 solutions was due to the presence of Fe^{3+} then a saturation level of 0.34 mg. would not have occurred, but instead a gradual approach to the Fe^{3+} plateau amount of 3.2 mg. would have been expected as the concentration of the filtered solutions was increased. If the Fe^{2+} and Fe^{3+} solutions used were first chelated with EDTA then the filter pads removed only traces of iron.

Fig. 2. Effect of the initial iron concentration of the medium on the specific growth rate of *Agrobacterium tumefaciens* (□), the maximum specific cellular glucoside 3-dehydrogenase activity (●) and the maximum accumulation of 3-ketosucrose in the culture supernatant (△).

Fig. 3. Uptake of Fe^{3+} and Fe^{2+} by Seitz filter pads (diameter 6 cm.): ○, from aqueous FeCl_3 solutions; ■, from acidified FeSO_4 solutions. Solutions each of 25 ml. but of different concentrations were filtered, and the iron in the filtrates was determined and compared with that in the original solutions.

DISCUSSION

The present study demonstrates that the iron requirement by the *Agrobacterium tumefaciens* strain for optimum glucoside 3-dehydrogenase activity and 3-ketosucrose production is much lower than that needed for an optimum growth rate. The passage of autoclaved medium through a Seitz filter pad proved an effective means of removing sufficient iron from a simple medium to obtain iron-limited growth. The medium prior to filtration contained no visible precipitate and seemingly the filter pads removed iron from solution by direct absorption.

The decrease in glucoside 3-dehydrogenase activity in iron-limited cultures does not necessarily indicate that this enzyme contains iron or is dependent on iron as a co-factor; van Beeumen & DeLey (1968), in fact, state that the enzyme is not a metallo-enzyme or a haem-protein. The fact that the optimum iron concentrations for growth rate and glucoside 3-dehydrogenase activity were not the same is evidence that 3-ketosucrose is not an essential intermediate in sucrose metabolism by *Agrobacterium tumefaciens*. There seems to be no clear evidence that 3-ketosucrose is an intermediate in sucrose metabolism; it may accumulate through interference with the regulation of the enzyme concerned.

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